Heterogeneity of hypoxia-mediated decrease in $I_{K(V)}$ and increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in pulmonary artery smooth muscle cells

Oleksandr Platoshyn, Ying Yu, Eun A Ko, Carmelle V. Remillard, and Jason X.-J. Yuan

Division of Pulmonary and Critical Care Medicine, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California

Submitted 3 October 2006; accepted in final form 23 May 2007

Heterogeneity of hypoxia-mediated decrease in $I_{K(V)}$ and increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 293: L402–L416, 2007. First published May 25, 2007; doi:10.1152/ajplung.00391.2006.—Hypoxic pulmonary vasoconstriction is caused by a rise in cytosolic Ca$^{2+}$ ([Ca$^{2+}]_{\text{cyt}}$) in pulmonary artery smooth muscle cells (PASMC) via multiple mechanisms. PASMC consist of heterogeneous phenotypes defined by contractility, proliferation, and apoptosis as well as by differences in expression and function of various genes. In rat PASMC, hypoxia-mediated decrease in voltage-gated K$^+$ current ($I_{K(V)}$) and increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ were not uniformly distributed in all PASMC tested. Acute hypoxia decreased $I_{K(V)}$ and increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in ~46% and ~53% of PASMC, respectively. Using combined techniques of single-cell RT-PCR and patch clamp, we show here that mRNA expression level of Kv1.5 in hypoxia-sensitive PASMC (in which hypoxia reduced $I_{K(V)}$) was much greater than in hypoxia-insensitive cells (in which hypoxia negligibly affected $I_{K(V)}$). These results demonstrate that 1) different PASMC express different K channel $\alpha$- and $\beta$-subunits, and 2) the sensitivity of a PASMC to acute hypoxia partially depends on the expression level of Kv1.5 channels; hypoxia reduces whole-cell $I_{K(V)}$ only in PASMC that express high level of Kv1.5. In addition, the acute hypoxia-mediated changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ also vary in different PASMC. Hypoxia increases $[\text{Ca}^{2+}]_{\text{cyt}}$ only in 34% of cells tested, and the different sensitivity of $[\text{Ca}^{2+}]_{\text{cyt}}$ to hypoxia was not related to the resting $[\text{Ca}^{2+}]_{\text{cyt}}$. An intrinsic mechanism within each individual cell may be involved in the heterogeneity of hypoxia-mediated effect on $[\text{Ca}^{2+}]_{\text{cyt}}$. These data suggest that the heterogeneity of PASMC may partially be related to different expression levels and functional sensitivity of K channels to hypoxia and to differences in intrinsic mechanisms involved in regulating $[\text{Ca}^{2+}]_{\text{cyt}}$.

Voltage-gated K$^+$ channel; Kv1.5; membrane potential; vascular smooth muscle cells

Hypoxic pulmonary vasoconstriction (HPV) is an important physiological mechanism that directs blood flow to well-ventilated areas in the lung to maximize oxygenation of the venous blood in pulmonary artery (12). Persistent or chronic exposure to hypoxia, however, causes pulmonary hypertension as a result of sustained pulmonary vasoconstriction and pulmonary vascular remodeling (49, 65, 76). Acute hypoxia-mediated pulmonary vasoconstriction occurs not only in isolated perfused lungs (46, 47) but also in isolated pulmonary arteries (8, 30, 34, 41, 42, 68, 88) and dissociated pulmonary artery smooth muscle cells (PASMC) (51, 86, 90). These observations suggest that hypoxia-mediated pulmonary vasconstriction is an intrinsic characteristic of PASMC and is caused by a unique mechanism specific to PASMC (1, 45, 49).

Acute hypoxia causes pulmonary vasoconstriction by raising cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}]_{\text{cyt}}$) in PASMC as a result of increased Ca$^{2+}$ influx and release (18, 22, 69, 79). One of the mechanisms involved in acute hypoxia-mediated increase in [Ca$^{2+}]_{\text{cyt}}$ is that hypoxia induces membrane depolarization by selectively reducing voltage-gated K$^+$ (Kv) channel activity (17, 63, 64, 86). Hypoxia also directly induces Ca$^{2+}$ mobilization from intracellular Ca$^{2+}$ stores (e.g., the sarcoplasmic reticulum, mitochondria, or lysosomes) to raise [Ca$^{2+}]_{\text{cyt}}$ and cause pulmonary vasoconstriction (21, 22, 34, 48, 53). It has been demonstrated that PASMC isolated from different segments of pulmonary arterial trees have different electrophysiological profiles, different responses to hypoxia, and different morphology (4, 6, 25). The hypoxia-induced increase in [Ca$^{2+}]_{\text{cyt}}$ and inhibition of Kv channels may thus vary among PASMC that are derived from different pulmonary arterial segments, alienated in different cell cycle phases, and differentiated into different phenotypes (e.g., contractile, proliferating, differentiating or quiescent phenotypes) or genotypes (e.g., due to somatic gene mutations).

Similar to intestinal smooth muscle and systemic vascular smooth muscle, PASMC may be electrophysiologically or functionally coupled to each other via gap junctions (13, 14, 23, 39, 70). Excitation (i.e., membrane depolarization or a rise in [Ca$^{2+}]_{\text{cyt}}$) of one PASMC would rapidly propagate to other PASMC via gap junction channels. Therefore, it would be more “efficient” that only a few PASMC in pulmonary arteries serve as “pacemaker-like” cells to trigger membrane depolarization or [Ca$^{2+}]_{\text{cyt}}$ increases in response to hypoxia; the excitation signal is then disseminated widely to the whole segment of pulmonary arteries to cause pulmonary vasoconstriction. Indeed, the pacemaker endothelial cells, located at capillary branch points in the pulmonary vasculature, have been shown to initiate Ca$^{2+}$ waves and generate [Ca$^{2+}]_{\text{cyt}}$ oscillations that can be propagated to adjacent “nonpacemaker” cells via gap junction channels (7, 38, 84). It seems that [Ca$^{2+}]_{\text{cyt}}$ regulation is homogeneous in lung endothelium and epithelium as well as in the vasculature. The initial Ca$^{2+}$ oscillation or transient is generated by a subclass of endothelial and epithelial cells, the pacemaker cells or the interstitial cells, and communicated to adjoining or neighboring cells via gap junctions (10, 11, 20, 24, 31, 32, 44, 84).

Heterogeneity of PASMC has been implicated in acute hypoxia-induced pulmonary vasoconstriction and chronic hy-
HETEROGENEITY OF HYPOXIC RESPONSE IN RAT PASMC

PASMC. Although cultured PASMC may undergo phenotypical changes, the hypoxia-mediated inhibitory effect on K+ channels augmenting the effect on \([Ca^{2+}]_t\) is comparable in freshly dissociated PASMC and primary cultured PASMC (4, 55, 59, 63, 64, 69, 72, 87). The cultured PASMC also maintain the capacity to contract in response to hypoxia through several passages in culture (51) and have similar whole-cell outward K+ currents relative to freshly dissociated cells (59). Furthermore, the response of cultured vascular smooth muscle cells to vasoactive agents is well preserved in cells cultured for various durations (9), suggesting that primary cultured smooth muscle cells retain their ability to respond to extracellular stimuli.

**Cell cycle analysis.** The cell cycle distribution of rat PASMC cultured in 0.2% FBS-DMEM was analyzed by flow cytometry. Briefly, cells were first cultured in 0.2% FBS-DMEM for 24 h. Then, the cells were trypsinized, washed one time with PBS, and fixed with 70% ethanol for at least 1 h at 4°C. The fixed cells were washed with PBS and incubated with a solution containing 0.05 mg/ml propidium iodine, 0.1% sodium citrate, and 50 μg/ml RNase A for 30 min at 4°C in the dark. The stained cells were analyzed by FACScanibur using CellQuest software (Becton Dickinson, Mountain View, CA) (77). The cell cycle was examined by measuring integrated green fluorescence (FL-1) and red fluorescence (FL-2) peaks using CellQuest software. Whole-cell K+ currents were recorded from PASMC with an Axopatch 1D amplifier and a DigiData 1200 interface (Axon Instruments) using conventional voltage-clamp techniques as described previously (88). Briefly, a coverslip with cells mounted was placed on a Plexiglas perfusion chamber on a Nikon inverted microscope, and cells on the coverslip were bathed in Ca2+-free physiological saline solution (PSS) containing (in mM) 141 NaCl, 4.7 KCl, 3.0 MgCl2, 10 HEPES, 1 EGTA, and 10 glucose (pH 7.4). The pipette (intracellular) solution contained (mM) 135 KCl, 4 MgCl2, 10 HEPES, 10 EGTA, and 5 disodium ATP (pH 7.2). All experiments were performed at room temperature (22–24°C).

Borosilicate patch pipettes (2–4 MΩ), which were fabricated on a model P-97 electrode puller (Sutter Instruments) and polished with a MF-63 microforge (Narishige Scientific Instruments Laboratories), were used to make high-resistance (2–4 GΩ) seals with the cell membrane for whole-cell voltage-clamp studies. After break-in, the averaged series resistance (Rs) was 21.3 ± 8.9 MΩ (mean ± SD, n = 27). A high membrane resistance (Rm, 2–3 GΩ) was recorded in most of the cells, and the Rm/Rs ratio was close to 1% (0.71–1.06%). However, the series resistance and membrane capacitance were compensated in all experiments using the patch clamp amplifier.

**Single-cell RT-PCR.** To determine the mRNA expression of endogenous Kv channels at the single-cell level, multiplex single-cell RT-PCR was performed according to a modified protocol (15). Briefly, after recording \(I_{K(V)}\) in a PASMC before, during, and after treatment with hypoxia, we carefully aspirated the whole cell into a collection pipette, which contained 12 μl of the pipette solution supplemented with 10 μM dNTPs and 0.5 U/μl RNase inhibitor. The content in the pipette was then expelled immediately into a 0.2-ml PCR tube that contained 8 μl of a solution composed of 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, 10 mM diithiothreitol, 1.25 mM oligo(dT), 0.5 mM dNTPs, and 5 U AMV reverse transcriptase XL. The reverse transcription (RT) was performed for 60 min at 42°C. Then, first-round PCR with 45 cycles was performed in the same tube by the addition of 80 μl of the premix PCR buffer containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, 20 mM of each sense and antisense primers (first primers) for all the genes of interest, and 5 U of Taq polymerase (RNA PCR kit, Takara). Two-microliner aliquots of the first-round PCR products were reamplified by the second-round PCR with 25–30 cycles, which was separately carried out using fully nested gene-specific primers (nested primers) for each target gene. Second-round PCR-amplified products were separated on 1.5% agarose gel and visualized with GelStar gel staining. To quantify the PCR products, β-actin was used as an internal control. The “surface plot” (or “3-D plot”) tool of the Image-Pro Plus (version 4.5) program was used to quantify the intensity of single-cell RT-PCR bands. The program created a three-dimensional representation of the band intensity, and the height of the three-dimensional images was used to

**MATERIALS AND METHODS**

**Cell preparation and culture.** Primary cultured PASMC were prepared from Sprague-Dawley rats according to the protocol described previously (86, 87). Briefly, the intrapulmonary arterial branches (3rd–4th divisions) were isolated in Hank’s solution (HBSS) comprised of Hank’s balanced salt (Irvine Scientific, Santa Ana, CA) supplemented with 14.98 mM N-2-hydroxethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1.7 mM NaHCO3, 0.4 mM MgSO4, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 0.02 mM CaCl2. The remaining smooth muscle was digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma) for 45 min at 37°C. During the enzymatic digestion, the smooth muscle tissue was triturated with a fire-polished Pasteur pipette to separate the cells from the bottom of centrifugation tube. The resulting pellets were resuspended in 2–3 ml 10% FBS-DMEM supplemented with 14.98 mM HEPES, 4.17 mM NaHCO3, 0.4 mM MgSO4, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 0.02 mM CaCl2. Parenchymal and adipose tissues were gently removed from the vessels under sterile conditions. The isolated artery was then incubated in HBSS with 1.5 mg/ml collagenase (Worthington) for 20 min. After the incubation, a thin layer of adventitia was carefully stripped off, and endothelium was removed by gently scratching the intimal surface. The remaining smooth muscle was digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma) for 45 min at 37°C. After the enzymatic digestion, the smooth muscle tissue was triturated 2–4 times with a fire-polished Pasteur pipette to speed digestion. The incubation mixture was then diluted 20-fold by adding 20% fetal bovine serum (FBS)-containing Dulbecco’s modified Eagle’s medium (DMEM, including 7 mM NaHCO3, 10 mM HEPES; pH 7.2) to stop digestion. The cell suspensions were centrifuged for 5 min at 1,500 rpm at room temperature, and the supernatant was removed. The resulting pellets were resuspended in 2–3 ml 10% FBS-DMEM and triturated with a fire-polished Pasteur pipette to separate the cells from the bottom of centrifugation tube. The cells were then plated onto 25-mm coverslips and cultured in 10% FBS-DMEM in a humidified incubator (at 37°C with 5% CO2) for 1 day. After the cells reached confluence, the culture medium was replaced with 0.2% FBS-DMEM and cultured in 0.2% FBS-DMEM for 48 h. Then, the cells were fixed with 70% ethanol for at least 1 h at 4°C. The fixed cells were washed with PBS and incubated with a solution containing 0.05 mg/ml propidium iodine, 0.1% sodium citrate, and 50 μg/ml RNase A for 30 min at 4°C in the dark. The stained cells were analyzed by FACScanibur using CellQuest software (Becton Dickinson, Mountain View, CA) (77). To determine the mRNA expression of endogenous Kv channels at the single-cell level, multiplex single-cell RT-PCR was performed according to a modified protocol (15). Briefly, after recording \(I_{K(V)}\) in a PASMC before, during, and after treatment with hypoxia, we carefully aspirated the whole cell into a collection pipette, which contained 12 μl of the pipette solution supplemented with 10 μM dNTPs and 0.5 U/μl RNase inhibitor. The content in the pipette was then expelled immediately into a 0.2-ml PCR tube that contained 8 μl of a solution composed of 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, 10 mM diithiothreitol, 1.25 mM oligo(dT), 0.5 mM dNTPs, and 5 U AMV reverse transcriptase XL. The reverse transcription (RT) was performed for 60 min at 42°C. Then, first-round PCR with 45 cycles was performed in the same tube by the addition of 80 μl of the premix PCR buffer containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, 20 mM of each sense and antisense primers (first primers) for all the genes of interest, and 5 U of Taq polymerase (RNA PCR kit, Takara). Two-microliner aliquots of the first-round PCR products were reamplified by the second-round PCR with 25–30 cycles, which was separately carried out using fully nested gene-specific primers (nested primers) for each target gene. Second-round PCR-amplified products were separated on 1.5% agarose gel and visualized with GelStar gel staining. To quantify the PCR products, β-actin was used as an internal control. The “surface plot” (or “3-D plot”) tool of the Image-Pro Plus (version 4.5) program was used to quantify the intensity of single-cell RT-PCR bands. The program created a three-dimensional representation of the band intensity, and the height of the three-dimensional images was used to...
compare the intensity among various PCR bands. The sense and antisense primers were specifically designed from the coding regions of rat Kv channel genes (Table 1). The fidelity and specificity of the sense and antisense oligonucleotides were examined using the BLAST program.

Measurement of \([\text{Ca}^{2+}]_{\text{cyt}}\). Cells on 25-mm coverslips were placed in a recording cell chamber on the stage of an inverted Nikon microscope (Eclipse/TE 200) with the TE-FM epifluorescence attachment. \([\text{Ca}^{2+}]_{\text{cyt}}\) in single PASMC was measured using the \(\text{Ca}^{2+}\)-sensitive fluorescent indicator, Calcium Green-1-AM (the acetoxymethyl ester of Calcium Green-1 dye). Cells were loaded with Calcium Green-1-AM, a cell-permeant acetoxymethyl ester form of fura-2 (fura-2-AM, 3 M). Calcium Green-1-AM and 1.5% DMSO (vol/vol). The Calcium Green-1-AM-loaded cells were then washed with PSS for 15 min to remove excess extracellular dye and DMSO (vol/vol). The Calcium Green-1-AM-loaded cells were then salt solution (PSS) containing 10 mM Calcium Green-1-AM and 1.5% DMSO (vol/vol). The Calcium Green-1-AM-loaded cells were then washed with PSS for 15 min to remove excess extracellular dye and to permit intracellular cleavage of the cell-permeant acetoxymethyl ester to the active Calcium Green-1-AM into active Calcium Green-1. In \(\text{Ca}^{2+}\)-free PSS, Calcium Green-1 was replaced by equimolar MgCl2, and 1 mM EGTA was added to chelate the residual Calcium Green-1. Excitation (488 nm) was provided by a mercury lamp. Fluorescence emission (\(>510\) nm) was collected using a x40 Nikon Plan Fluor objective and an intensifier. The cell “Ca2+” signals emitted from the cells were monitored continuously using an IBM-compatible computer for later analysis. Although we may refer to the images as “Ca2+” images, all the images are simply of \(\text{Ca}^{2+}\)-dependent Calcium Green-1 fluorescence images.

In some experiments, \([\text{Ca}^{2+}]_{\text{cyt}}\) was measured using the dual-wavelength \(\text{Ca}^{2+}\)-sensitive indicator, fura-2. Cells were loaded with the membrane-permeable acetoxymethyl ester form of fura-2 (fura-2-AM, 3 \(\mu\)M) for 30 min in the dark at room temperature (22–24°C). The fura-2-AM-loaded cells were then superfused with PSS for 20 min at 34°C to wash away extracellular dye and to permit intracellular cleavage of fura-2-AM to active fura-2 by cytoplasmic esterases. Fura-2 fluorescence from the cells and background fluorescence were collected at 32°C using Nikon UV-Fluor objectives. The fluorescence signals emitted from the cells were monitored continuously using an fluorescence microscopy system (Intracellular Imaging) and recorded on an IBM-compatible computer for later analysis. \([\text{Ca}^{2+}]_{\text{cyt}}\) was calculated from the fura-2 fluorescence emission at 340 nm and 380 nm (F340/F380) using a ratiometric method based on the following equation (28): \([\text{Ca}^{2+}]_{\text{cyt}} = K_d \times (S_{\text{max}} - S_{\text{cur}}) / ((R - R_{\text{min}}) + (R_{\text{max}} - R))\), where \(K_d\) (225 mM) is the dissociation constant for \(\text{Ca}^{2+}\); \(S_{\text{cur}}\) and \(S_{\text{max}}\) are emission fluorescence values at 360-nm excitation in the presence of EGTA and Triton X-100, respectively; \(R\) is the measured fluorescence ratio; and \(R_{\text{min}}\) and \(R_{\text{max}}\) are minimal and maximal ratios, respectively. In all experiments, multiple cells were imaged in a single field, and one arbitrarily chosen peripheral cytosolic area from each cell was spatially averaged.

Hypoxic treatment. Normoxic conditions were established by bubbling the superfusion solution with room air to achieve oxygen tension (\(P_O_2\)) ranging from 140 to 149 mmHg at 24°C. Acute hypoxia was established by dissolving 0.8 mM sodium dithionite (\(Na_2S_2O_4\), Sigma), an oxygen scavenger that combines with \(O_2\) and decreases \(P_O_2\) in solution, in the extracellular solution to achieve a \(P_O_2\) ranging from 22 to 40 mmHg. An oxygen electrode (Microelectrodes) was positioned in the cell chamber on the microscope stage to continuously monitor the \(P_O_2\). Sodium dithionite had no effect on the activity of endogenous Kv channels in native PASMC (61, 86) or exogenous Kv1.5 channels in PASMC transiently transfected with the KCNQ5 gene (61), unless accompanied by a reduction in \(P_O_2\). Rigorously bubbling the \(Na_2S_2O_4\) (0.8 mM)-containing solution with room air for 20–30 min increased the \(P_O_2\) to \(\sim 145\) mmHg (61, 86).

Statistical analysis. The composite data are expressed as means ± SE. Statistical analysis was performed using the unpaired Student’s t-test or one-way analysis of variance (ANOVA) where appropriate. Differences were considered to be significant when \(P < 0.05\). RESULTS

Contractile phenotype of the cells used for electrophysiological and fluorescence microscopy experiments. The rat PASMC incubated in 0.2% FBS-DMEM (Fig. 1Aa) show similar morphology compared with the cells cultured in 10% FBS-DMEM (data not shown). The flow cytometry data indi-

<table>
<thead>
<tr>
<th>Standard Names with Accession No.*</th>
<th>Size, bp</th>
<th>Predicted Sense and Antisense</th>
<th>Location, nt</th>
<th>Gene, chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.1 (KCNJ1), NM_0173095</td>
<td>594 bp</td>
<td>Sense 5'-ATCTCAGCTTGGCAAGCCAGGAG-3'</td>
<td>1151–1178</td>
<td>4q24</td>
</tr>
<tr>
<td>Nested primers (298 bp)</td>
<td></td>
<td>Antisense 5'-GTCGTTTTTTTAAAACTGAGGTTT-3'</td>
<td>1724–1744</td>
<td></td>
</tr>
<tr>
<td>Kv1.2 (KCNJ2), NM_008417</td>
<td>295 bp</td>
<td>Sense 5'-ATACGAGGATACAGAGG-3'</td>
<td>1934–1952</td>
<td>2q41</td>
</tr>
<tr>
<td>Nested primers (163 bp)</td>
<td></td>
<td>Antisense 5'-ATATTCTTGTGGCTAATCAG-3'</td>
<td>2209–2228</td>
<td></td>
</tr>
<tr>
<td>Kv1.5 (KCNJ5), M27158</td>
<td>267 bp</td>
<td>Sense 5'-ATCGTGCGGTTCATCGTC-3'</td>
<td>2088–2108</td>
<td>2q43</td>
</tr>
<tr>
<td>Nested primers (196 bp)</td>
<td></td>
<td>Antisense 5'-AGATCTGTGGCAGGTGAGGC-3'</td>
<td>2080–2099</td>
<td></td>
</tr>
<tr>
<td>Kv6.3 (KCNJ6), AB070605</td>
<td>422 bp</td>
<td>Sense 5'-ATCGTGCGGTTCATCGTC-3'</td>
<td>2209–2228</td>
<td></td>
</tr>
<tr>
<td>Nested primers (221 bp)</td>
<td></td>
<td>Antisense 5'-AGATCTGTGGCAGGTGAGGC-3'</td>
<td>2080–2099</td>
<td></td>
</tr>
<tr>
<td>Kvβ1.1 (KCNJ1), NM_017303</td>
<td>194 bp</td>
<td>Sense 5'-GCAACAGCCGCTACACAGAA-3'</td>
<td>1826–1845</td>
<td>2q31</td>
</tr>
<tr>
<td>Nested primers (150 bp)</td>
<td></td>
<td>Antisense 5'-AGATCTGTGGCAGGTGAGGC-3'</td>
<td>2000–2019</td>
<td></td>
</tr>
<tr>
<td>β-Actin, V01217</td>
<td>414 bp</td>
<td>Sense 5'-CTCGAAGTACGTACCTGGCAGAA-3'</td>
<td>2528–2549</td>
<td>2q43</td>
</tr>
<tr>
<td>Nested primers (244 bp)</td>
<td></td>
<td>Antisense 5'-AGATCTGTGGCAGGTGAGGC-3'</td>
<td>3129–3150</td>
<td></td>
</tr>
</tbody>
</table>

*The accession numbers are the GenBank numbers for the sequence used in designing the primer.

AJP-Lung Cell Mol Physiol • VOL 293 • AUGUST 2007 • www.ajplung.org
cated that 73.1% of the cells cultured in 0.2% FBS-DMEM were in G0/G1 phases, while 2.3% were in S phase and 21.3% in G2/M phases (Fig. 1). Furthermore, the cells cultured onto coverslips also maintain contractility in response to high K+ (40 mM K+). As shown in Fig. 1, raising extracellular [K+] from 4.7 mM to 40 mM reversibly caused a 12-μm shortening (Fig. 1B), or a 30–40% decrease (Fig. 1C), of the cell length. Blockade of voltage-gated Ca2+ channels with 10 μM nifedipine almost abolished the 40 mM K+-induced cell shortening (Fig. 1A). These results suggest that most of the cells used for the following patch clamp and fluorescence microscopy experiments (cultured in 0.2% FBS-DMEM) were in G0/G1 phases and maintained their contractile phenotype.

Whole-cell outward K+ currents in rat PASMC. In PASMC dialyzed with 135 mM K+-containing and 5 mM ATP-containing solution and superfused with Ca2+-free solution, depolarization from a holding potential of −70 mV to a series of test potentials ranging from −60 mV to +80 mV elicited only outward K+ currents (Fig. 2A). The currents at positive potentials (e.g., +80 mV) appeared to include two components based on their inactivation kinetics: a slowly inactivating component and a sustained noninactivating component (Fig. 2Aa). The voltage threshold for activating outward K+ currents ranged from −60 to −40 mV (Fig. 2Ab and c). The currents were rapidly activated with a time constant of 1.17 ms and rapidly deactivated with a time constant of 0.93 ms (Fig. 2B and C). The currents were sensitive to the Kv channel blocker, 4-amipopyridine (4-AP), but insensitive to the Ca2+-activated K+ (KCa) channel blocker, charybdotoxin (50 nM), and the ATP-sensitive K+ (KATP) channel blocker, glibenclamide (10 μM) (data not shown). These results indicate that the outward K+ currents in PASMC under these conditions were mainly Kv currents; the contribution of KCa and KATP currents was minimized in these experiments because of ATP (5 mM) and EGTA (10 mM) in the pipette (intracellular) solution and Ca2+-free (plus 1 mM EGTA) bath (extracellular) solution.

Amplitude and kinetics of whole-cell IK(V) in PASMC partially depend on the expression level of various Kv channel α-subunits (the functional pore-forming subunits), β-subunits (the regulatory subunits), and γ-subunits (the electrically silent pore-forming subunits) (5, 16, 33, 57, 59, 62). In this study, we used single-cell RT-PCR and patch clamp techniques to determine (or correlate) the mRNA expression level of certain Kv channel subunits in the cell from which we recorded whole-cell IK(V). As shown in Fig. 3, the amount of RT-PCR product for Kv1.5, for example, in a PASMC amplified by the nested primers was linearly increased between 25 and 30 cycles. This result indicates that 27 cycles is an optimal condition with which to amplify and semiquantitatively compare the mRNA level of Kv channel transcripts in single cells.

![Fig. 1. Cell cycle distribution and contractile response to high K+ of rat pulmonary artery smooth muscle cells (PASMC). A: a representative phase-contrast image (a) showing rat PASMC cultured in 0.2% FBS-DMEM and a histogram (b) showing the distribution of the cells cultured in 0.2% FBS-DMEM in different cell cycle phases. B: a representative time course curve showing the change in cell length (measured by the longitudinal line across the cell, inset) in a PASMC before, during, and after superfusion with 40 mM K+-containing solution. C: averaged data (means ± SE, n = 8) showing the relative change of cell length 580 s (9.7 min) and 980 s (16.3 min) after superfusion with 40 mM K+-containing solution as well as −1,110 s (18.5 min, or at 34.8 min shown in C) 1,460 s (24.3 min, or at 40.6 min shown in C) after washout with regular PSS (containing 4.7 mM K+). **P < 0.01 vs. control length (open bar). D: time course of changes in cell length in PASMC superfused with 40 mM K+-containing PSS in the presence (solid circles) or absence (open circles) of 10 μM nifedipine. Data are means ± SE (n = 11 cells for each data point; P < 0.001 between the two curves).](http://ajplung.physiology.org/)
Acute hypoxia reduces whole-cell $I_{K(V)}$ only in a small portion of PASMC. In cultured PASMC prepared from the same pulmonary arterial branches of rats, reducing $P_{O_2}$ from $147 \pm 1$ to $40 \pm 3$ mmHg significantly decreased whole-cell $I_{K(V)}$ in some cells, but negligibly affected $I_{K(V)}$ in others (Fig. 4). The currents in hypoxia-sensitive cells (shown in red) and the currents in hypoxia-insensitive cells (shown in blue) were, however, not significantly different. To examine the possibility that the different response of whole-cell $I_{K(V)}$ to acute hypoxia in various PASMC may be attributed to the distinctive expression of different Kv channel subunits, we measured mRNA levels of certain subunits in the same cell in which $I_{K(V)}$ was recorded and challenged with acute hypoxia. Specifically, after recording $I_{K(V)}$ in each PASMC before, during, and after acute exposure to hypoxia, the cell was collected by a large pipette or the recording pipette to conduct single-cell RT-PCR experiments.

Correlation of Kv1.5 mRNA level with hypoxia-induced reduction of $I_{K(V)}$. Using combined techniques of patch clamp and single-cell RT-PCR, we identified that the mRNA level of Kv1.5, a functional pore-forming $\alpha$-subunit, was much higher in hypoxia-sensitive PASMC than in hypoxia-insensitive PASMC (Fig. 4, Aa and Ba, top; as well as Ab and Bb). However, the mRNA expression levels of other $\alpha$-subunits ($Kv1.1$, $Kv1.2$), $\beta$-subunits ($Kv\beta1.1$), and $\gamma$-subunits ($Kv6.3$), as well as a conserved internal control, $\beta$-actin, were all comparable between hypoxia-sensitive and hypoxia-insensitive cells (Fig. 4, A–C, a, top; and b). The three-dimensional images (Fig. 4, b panels), constructed from the intensity data of the single-cell RT-PCR bands (Fig. 4, a panels, top), further illustrated the difference of Kv1.5 mRNA expression between hypoxia-sensitive and hypoxia-insensitive PASMC. These results indicate that the mRNA expression level of Kv1.5 correlates with the hypoxia sensitivity of whole-cell $I_{K(V)}$ in rat PASMC (i.e., $I_{K(V)}$ recorded in cells with high expression level of Kv1.5 are sensitive to acute hypoxia). We previously reported that acute hypoxia significantly reduced $I_{K(V)}$ in all rat PASMC overexpressing the human Kv1.5 ($KCNAT5$) gene (61), further indicating that Kv1.5 channel is involved in forming hypoxia-sensitive Kv channels in PASMC.

These results direct us to speculate that rat pulmonary artery contains phenotypically distinctive PASMC; the phenotypic difference (e.g., responsiveness to hypoxia) among individual PASMC is maintained in culture (and in generations). To determine whether the biophysical properties of Kv channels are phenotypically different between the hypoxia-sensitive and hypoxia-insensitive PASMC, we compared the amplitude and kinetics of whole-cell $I_{K(V)}$ between cells in which the currents were inhibited by acute hypoxia and cells in which the currents were negligibly affected by acute hypoxia.

Comparison of Kv current kinetics between hypoxia-sensitive and hypoxia-insensitive PASMC. Whole-cell $I_{K(V)}$ exhibited similar amplitude, activation and deactivation kinetics, and activation threshold in hypoxia-sensitive PASMC (Fig. 5A) and hypoxia-insensitive cells (Fig. 5B). Acute hypoxia reduced the amplitude and current density of $I_{K(V)}$ at $+80$ mV from $656 \pm 74$ to $282 \pm 45$ pA and from $62 \pm 2$ to $25 \pm 2$ pA/pF ($n = 16, P < 0.001$), respectively, in hypoxia-sensitive PASMC. The percent decrease of the amplitude of $I_{K(V)}$ elicited by different test potentials ranged from $34 \pm 19\%$ at $-20$ mV to $100\%$ at $+80$ mV, as illustrated in Fig. 5B.
mV to 58 ± 4% at +80 mV (Fig. 5Ab). The inhibitory effect of acute hypoxia on \(I_{K(V)}\) was completely reversible (Fig. 5A).

In hypoxia-sensitive PASMC, however, acute hypoxia did not affect kinetics of activation (\(\tau_{\text{act}}\)), inactivation (\(\tau_{\text{inact}}\)), and deactivation (\(\tau_{\text{deact}}\)) of whole-cell \(I_{K(V)}\) (Fig. 5Ac). In hypoxia-insensitive cells, acute hypoxia neither affected the amplitude and current density of \(\text{Kv}\) currents nor altered the kinetics of activation, inactivation, and deactivation (Fig. 5B).

By comparing the currents recorded in hypoxia-sensitive and hypoxia-insensitive PASMC under normoxic conditions, we observed that the amplitude and density of \(I_{K(V)}\) at +80 mV were similar (Fig. 6Aa); the current amplitude at −40 mV, however, was ~35% higher in hypoxia-insensitive cells (Fig. 6Ab, left; \(P < 0.05\)), although the current density at −40 mV was comparable (Fig. 6Ab, right). Furthermore, the \(\text{Kv}\) currents at +80 mV in hypoxia-sensitive cells appeared to inactivate more rapidly than those in hypoxia-insensitive cells (Fig. 5, Ac and Bc, middle); \(\tau_{\text{inact}}\) was 76 and 113 ms, respectively. These results indicate that the amplitude and current density of whole-cell \(I_{K(V)}\) are readily comparable at high test potentials (e.g., +80 mV) in hypoxia-sensitive and hypoxia-insensitive cells, but the current amplitude at the negative potential (e.g., −40 mV) close to the resting membrane potential is slightly higher in hypoxia-sensitive cells than in hypoxia-insensitive cells. The difference between these two PASMC phenotypes seems to figure predominantly in the responsiveness of \(I_{K(V)}\) to hypoxia.

In addition to recording and comparing the amplitude and density of \(I_{K(V)}\) in hypoxia-sensitive and hypoxia-insensitive cells, we also measured mRNA expression levels of \(\text{Kv}\) channel α- (Kv1.5), β- (Kvβ1.1), and γ-subunits (Kv6.3) in each of the cells using single-cell RT-PCR (Fig. 6B). In these experiments, we used 27 cycles to amplify cDNA products using the P/4 protocol. Horizontal and vertical bars shown in each record denote 100 ms and 100 pA, respectively. β-Actin (244 bp) was used as an internal control for the single-cell RT-PCR experiments. M, 100-bp DNA ladder. The scale bar shown inCb denotes the relative intensity of the PCR bands in arbitrary units.
in Fig. 6B, the mRNA expression level of Kv1.5 channel was much higher in hypoxia-sensitive PASMC than in hypoxia-insensitive cells (also see Fig. 4), whereas mRNA levels of Kv1.1.1 (a cytoplasmic regulatory subunit), Kv6.3 (an electrically silent subunit), and β-actin were all comparable. The association of high expression level of Kv1.5 with the sensitivity of $I_{K(V)}$ to acute hypoxia implies that the Kv1.5 channel may be an important subunit in forming functional Kv channels in hypoxia-sensitive PASMC and that the Kv1.5 channel, by forming homotetrameric or heterotetrameric channels with other subunits, may be an important target for hypoxia to reduce $I_{K(V)}$ in PASMC.

Acute hypoxia induces Ca$^{2+}$ mobilization in one-third of PASMC tested. In addition to triggering membrane depolarization (by inhibiting K$^+$ channel activity) and subsequently increasing Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels (17, 63, 64, 85, 86), acute hypoxia has also been demonstrated to cause Ca$^{2+}$ release from intracellular stores in PASMC (21, 22, 34, 48, 53, 63, 79). The heterogeneity of PASMC in response to acute hypoxia is not only indicated by changes in $I_{K(V)}$ but also manifested by changes in Ca$^{2+}$ mobilization from intracellular stores. As shown in Fig. 7, exposure of PASMC bathed in Ca$^{2+}$-free solution to acute hypoxia (PO$_2$ = 35 ± 2 mmHg) increased [Ca$^{2+}$]$_{cyt}$ in a fraction of the cells tested. Out of 358 cells (at the same passage) we examined in 7 different experiments, we found that 34% of cells responded to hypoxia, whereas 66% of the cells were not affected by hypoxia at all (Fig. 8, A and B). There was no significant morphological difference between cells in which acute hypoxia induced Ca$^{2+}$ release and cells in which hypoxia had no effect on [Ca$^{2+}$]$_{cyt}$ in the absence of extracellular Ca$^{2+}$. As shown in Fig. 8C, although the cell area varies dramatically among different cells (ranging from 2,000 to 12,000 $\mu$m$^2$), the averaged cell area of hypoxia-sensitive cells (4,599.9 ± 156.1 $\mu$m$^2$, n = 123 cells) was actually very similar to that of hypoxia-insensitive cells (4,527.7 ± 130.9 $\mu$m$^2$, n = 235 cells; P = 0.736).

Among hypoxia-sensitive cells, the kinetics of hypoxia-induced increase in [Ca$^{2+}$]$_{cyt}$ due to Ca$^{2+}$ mobilization from...
intracellular stores seems to be different as well. Upon exposure to hypoxia, some cells exhibited a rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, whereas in other cells there seemed to be a delay before $\text{Ca}^{2+}$ was mobilized from intracellular stores (Fig. 7). Furthermore, some cells exhibited $\text{Ca}^{2+}$ oscillations, whereas other cells only showed a rapid (or delayed) single transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 7B). The amplitude of the hypoxia-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ also varied markedly among hypoxia-sensitive cells (Fig. 8D, left). The time from introduction of hypoxia to the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ ranged from <15 s to 3.5 min (Fig. 8D, right); the average time-to-peak for hypoxia to induce $\text{Ca}^{2+}$ release in hypoxia-sensitive PASMC was $1.27 \pm 0.06$ min. These data indicate that acute hypoxia induces $\text{Ca}^{2+}$ release from intracellular stores in about one-third of PASMC, with differences in amplitude and time course. The hypoxia-sensitive cells, in which acute hypoxia increases $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of extracellular $\text{Ca}^{2+}$, seem to be morphologically similar to the hypoxia-insensitive cells, in which hypoxia had no effect on $[\text{Ca}^{2+}]_{\text{cyt}}$.

Vascular smooth muscle cells (including human and animal PASMC) have been well documented to have at least two functionally distinctive intracellular stores: an inositol 1,4,5-trisphosphate (IP$_3$)-sensitive or IP$_3$-releasable store that can be depleted by cyclopiazonic acid (CPA) and thapsigargin, and a ryanodine-sensitive store that is sensitive to caffeine (26, 35, 78). The next set of experiments was designed to determine whether pretreatment of PASMC with CPA or depletion of the IP$_3$-sensitive intracellular stores affected hypoxia-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to $\text{Ca}^{2+}$ release.

Passive depletion of IP$_3$-sensitive $\text{Ca}^{2+}$ stores with CPA abolished acute hypoxia-mediated $\text{Ca}^{2+}$ mobilization in PASMC. By activating the P$_2$ purinoceptor (a G protein-coupled receptor), ATP (2 mM) increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC (Fig. 9A) by causing $\text{Ca}^{2+}$ release from IP$_3$-sensitive intracellular stores and $\text{Ca}^{2+}$ influx through receptor- and store-operated $\text{Ca}^{2+}$ channels, as we showed previously (91). As shown in Fig. 9, treatment of PASMC with CPA in the absence of extracellular $\text{Ca}^{2+}$ induced a slow $[\text{Ca}^{2+}]_{\text{cyt}}$ rise due to $\text{Ca}^{2+}$

Fig. 7. Acute hypoxia increases cytosolic $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_{\text{cyt}}$) by inducing $\text{Ca}^{2+}$ release from intracellular stores in some PASMC. A: representative fluorescence images showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before (normoxia) and after hypoxic challenges for 40–160 s in PASMC superfused with $\text{Ca}^{2+}$-free solution. B: time course of the $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in 12 PASMC (labeled 1–12 in the image shown in the left). Acute hypoxia induces an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to $\text{Ca}^{2+}$ release in 7 PASMC tested (cells 1, 2, 4, 5, 6, 7, and 10; red), but has no effect on 5 PASMC (cells 3, 8, 9, 11, and 12; blue).
leakage from the IP3-sensitive stores. Approximately 10–15 min after CPA treatment, application of ATP failed to raise \([Ca^{2+}]_{\text{cyt}}\) in PASMC superfused with Ca2+-free solution, indicating that the IP3-sensitive store was readily depleted (Fig. 8A). Under these conditions, restoration of extracellular Ca2+ caused a large increase in \([Ca^{2+}]_{\text{cyt}}\) due apparently to Ca2+ influx through store-operated Ca2+ channels (27, 40, 52).

These data indicate that 10- to 15-min treatment of PASMC with CPA would virtually deplete Ca2+ in the IP3-sensitive SR.

In PASMC pretreated with 10 μM CPA, acute hypoxia failed to induce increase in \([Ca^{2+}]_{\text{cyt}}\) in any PASMC (Fig. 10, A and B). The averaged data from 61 cells tested show that short-term (30 s) and long-term (120 s) exposure to hypoxia negligibly altered \([Ca^{2+}]_{\text{cyt}}\) in PASMC pretreated with CPA for 15 min (Fig. 10C). These experiments suggest that the predominant recourse of hypoxia-induced Ca2+ release in hypoxia-sensitive PASMC is CPA-sensitive stores. Cell viability was examined using a maximal dose of ATP (2 mM). As shown in Fig. 11, extracellular application of 2 mM ATP increased \([Ca^{2+}]_{\text{cyt}}\) in almost all PASMC examined. The uniform response of PASMC to ATP indicates that hypoxia-insensitive cells were not dysfunctional or had intracellular stores depleted before hypoxic challenge.

**DISCUSSION**

One of the important mechanisms by which acute hypoxia causes pulmonary vasoconstriction is to increase \([Ca^{2+}]_{\text{cyt}}\) in PASMC by inducing Ca2+ release from intracellular stores and Ca2+ influx through plasmalemmal Ca2+ channels (21, 22, 30, 34, 41, 63, 69, 85). Hypoxia selectively inhibits K+ channel activity in PASMC (compared with systemic arterial smooth
appears not to be correlated with cell morphology (e.g., cell surface area and cell capacitance). These results imply that cultured PASMC exhibit (and/or maintain) heterogeneous responsiveness or sensitivity to hypoxia. Whether acute hypoxia reduces $I_{K(V)}$ in a PASMC is partially dependent of the expression level of Kv1.5; i.e., hypoxia reduces whole-cell $I_{K(V)}$ only in PASMC that expressed high levels of Kv1.5. In addition, whether acute hypoxia induces increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ seems to be dependent of the function and/or capacity of CPA-sensitive (and IP$_3$-sensitive) intracellular stores; i.e., hypoxia induces Ca$^{2+}$ release only in PASMC in which the IP$_3$-sensitive sarcoplasmic reticulum is full of Ca$^{2+}$.

Role of Kv1.5 channels in hypoxia-induced decrease in whole-cell $I_{K(V)}$. Acute hypoxia reduces $I_{K(V)}$ only in PASMC, but not in systemic (e.g., renal and mesenteric) arterial smooth muscle cells (64, 86). However, Kv1.5 channel is highly expressed in both pulmonary and systemic arterial smooth muscle cells (5, 6, 19, 60, 89). This indicates that the Kv1.5 channel is probably not the oxygen sensor in PASMC to initiate hypoxia-induced membrane depolarization and HPV but is an important effector that is regulated by a specific oxygen-sensing mechanism intrinsic to PASMC. Overexpression of Kv1.5 in PASMC confers hypoxic sensitivity to all PASMC, but, very importantly, overexpression of Kv1.5 does not confer hypoxic sensitivity to mesenteric arterial SMC, HEK-293, and COS-7 cells (61). These data suggest that Kv1.5 (as a homo- or heterotetramer) is a critical target of the oxygen-sensing mechanism in PASMC to exert functional effects (e.g., membrane depolarization, opening of voltage-dependent Ca$^{2+}$ channels, and increase in $[\text{Ca}^{2+}]_{\text{cyt}}$). The selectivity of HPV or hypoxia-induced inhibition of $I_{K(V)}$ in PASMC is thus achieved by a series of events comprised of an initial oxygen-sensing mechanism and a subsequent selection of downstream targets or effectors (e.g., which Kv channel subunit).

Heterogeneity of PASMC in response to acute hypoxia: role of $K(V)$ channels. The difference between hypoxia-sensitive and hypoxia-insensitive PASMC, as shown in this study, is partially manifested by hypoxia-mediated changes in $I_{K(V)}$. However, the biophysical properties of Kv channels appear to be comparable. The cells in which Kv1.5 is a major $\alpha$-subunit to form functional Kv channels are sensitive to acute hypoxia, whereas the cells in which Kv1.5 expression is low are insensitive to hypoxia. These data suggest an important concept: total Kv currents, regardless of the channel subunits that contribute to the currents, regulate (and maintain) the resting membrane potential (85, 86), while the composition of Kv channel $\alpha/\beta$-subunits that contribute to the whole-cell $I_{K(V)}$ determines (or participates in determining) the sensitivity or responsiveness of $I_{K(V)}$ to hypoxia. The hypoxia-sensitive cells use the Kv1.5 channel subunit, which is polymerized homogeneously or heterogeneously with other $\alpha/\beta$-subunits (e.g., Kv1.2 and Kv9.3) (5, 6, 33, 58), as a specific effector (or target) to inhibit the Kv channel function, causes membrane depolarization, enhances Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels, and increases $[\text{Ca}^{2+}]_{\text{cyt}}$. The membrane depolarization and increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca$^{2+}$ influx then propagate to hypoxia-insensitive cells via gap junction channels to, eventually, induce a uniformly distributed contraction in the pulmonary artery.
Differential expression of ion channels in different PASMC is not restricted to Kv channels (this study and see Ref. 4). Cornfield et al. (66, 67) have also provided compelling evidence that the large-conductance \( \text{Ca}^{2+} \)-activated \( \text{K} \) or maxi \( \text{K} \) channels are developmentally regulated and distinctively distributed (in terms of activity and expression level) in different PASMC. Hypoxia regulates maxi \( \text{K} \) channel activity via an adjacent heme oxygenase (2, 36, 37, 82). What types of \( \text{K} \) channel subunits contribute to whole-cell \( \text{K} \) currents seem to be very important to the cells, because it determines, at least, the sensitivity of \( I_{\text{K(V)}} \) to hypoxia. In addition to Kv1.5, other Kv channel \( \alpha \)- and \( \beta \)-subunits as well as two-pore/four-domain \( \text{K} \) (\( K_{\text{TP}} \) or \( K_{\text{ATP}} \)) channels have also been demonstrated to participate in forming oxygen-sensitive Kv channels in PASMC (5, 6, 16, 29, 55, 56, 58). It is unknown whether \( \text{K} \) channels genetically belonging to different subfamilies (e.g., Kv, \( K_{\text{Ca}} \), \( K_{\text{F}} \), and \( K_{\text{ATP}} \) channels) can functionally and physically interact with each other in lipid raft microdomains (e.g., caveolae) or in cytoplasmic compartmentalized vicinity in PASMC to form a complex that includes the sensor(s) (e.g., mitochondria, NADPH oxidase, polyhydroxylase) and the effector (e.g., various \( \text{K} \) and \( \text{Ca}^{2+} \) channels) (43, 50).

**Heterogeneity of PASMC in response to acute hypoxia: role of intracellular \( \text{Ca}^{2+} \) stores.** The precise mechanism involved in hypoxia-induced \( \text{Ca}^{2+} \) release is unclear; however, it may be related to 1) changes in reactive oxygen species (ROS) produced in the mitochondria and the cytoplasm (3, 80), 2) direct activation of different IP\(_3\) and ryanodine receptors on the SR membrane (21, 34, 48, 81), and 3) inhibition of the \( \text{Ca}^{2+} \)-Mg\(^{2+}\)-ATPase on the SR membrane (SERCA) (69). The same concept applied to Kv channels as mentioned above can also be used to explain the difference of hypoxia-induced \( \text{Ca}^{2+} \) mobilization in hypoxia-sensitive and hypoxia-insensitive PASMC. That is, the location of mitochondrial and cytoplasmic NADPH oxidases that are stimulated by hypoxia to produce ROS, the composition of IP\(_3\) and ryanodine receptor (and SERCA)
isoforms on the SR membrane, and the functional interaction and physical location of the oxygen sensor(s) and effector (e.g., the Ca\(^{2+}\) release channels on the SR) may differ in hypoxia-sensitive and hypoxia-insensitive PASMCL

Sham and his colleagues (83) have demonstrated that mRNA and protein expression levels of different ryanodine receptor isoforms are different among vascular smooth muscle cells, while location of different ryanodine receptor isoforms also differ between systemic and pulmonary vascular smooth muscle cells. The data on kinetics of hypoxia-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in hypoxia-sensitive PASMCL indicate that hypoxia-induced Ca\(^{2+}\) release can be delayed in some cells, further suggesting that intracellular Ca\(^{2+}\) stores may have different sensitivity to hypoxia because different mechanisms (e.g., mitochondrial vs. cytoplasmic production of ROS; local and global changes in redox status) are used to trigger Ca\(^{2+}\) mobilization.

One of the important questions our study is unable to answer is whether the cells showing hypoxia sensitivity in terms of changes in \(I_{K(V)}\) are the same cells that have sensitivity to hypoxia in terms of changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) (or Ca\(^{2+}\) mobilization). It would be ideal to use combined techniques of patch clamp and fluorescence microscopy to measure \(I_{K(V)}\) and \([\text{Ca}^{2+}]_{\text{cyt}}\), simultaneously, and then to determine the effect of acute hypoxia. We are currently not able to simultaneously measure \(I_{K(V)}\) and \([\text{Ca}^{2+}]_{\text{cyt}}\) in the same cell and correlate with mRNA expression of different Kv channel subunits as well as different ryanodine and IP\(_3\) receptors. However, an important concept is that, regardless of the precise mechanism initially involved in sensing hypoxia and how hypoxia induces membrane depolarization and Ca\(^{2+}\) mobilization, it is unnecessary for all contractile PASMCL to be hypoxia sensitive, or to have (or establish) a unique and selective hypoxia “sensor” or oxygen-sensing system.

**Heterogeneity of PASMC in response to acute hypoxia: role of cell phenotype.** Experiments in vivo and in vitro have shown that the media of systemic and pulmonary arteries contains phenotypically different smooth muscle cells, such as contractile (differentiated), synthetic, and proliferative SMC (25, 71, 75). Flow cytometry data also demonstrate that cultured PASMCL isolated from the same segment of pulmonary arterial branch are unevenly distributed into difference phases of the cell cycle (e.g., G0, G1, S, G2, or M phase). Therefore, the difference between hypoxia-sensitive and hypoxia-insensitive PASMCL, with regard to the changes in \(I_{K(V)}\) and Ca\(^{2+}\) mobilization to hypoxia, is probably because the cells in culture are 1) distributed in different cell cycle phases, 2) alienated into different phenotypes, 3) developed into different genotypes due to somatic mutations of genes, and 4) transformed from differentiated state to dedifferentiated state. The results from this study also indicate that 1) cells from the same segment of pulmonary arterial tree may have different response to hypoxia and 2) the different oxygen sensitivity in cells isolated from the same segment can be maintained for many generations or passages (i.e., if the differences of \(I_{K(V)}\) and \([\text{Ca}^{2+}]_{\text{cyt}}\) in response to hypoxia are due to different “parent” cells isolated from different segments of pulmonary artery, then the “daugh-

---

**Fig. 11.** ATP increases \([\text{Ca}^{2+}]_{\text{cyt}}\) by inducing Ca\(^{2+}\) release from intracellular stores in almost all PASMCL. Time course of the \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in 14 PASMCL (A) labeled as 1–14 (B). ATP induces an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to Ca\(^{2+}\) release in most of the cells tested.
ter” cells in culture still maintain the unique property, i.e., the sensitivity to hypoxia).

The report from Archer et al. (4), obtained from freshly dissociated PASMC from different pulmonary artery segments, is different from our results obtained from cultured PASMC. Their data show that PASMC from different segments of pulmonary artery (e.g., conduit vs. small intrapulmonary arteries) exhibit different activity of Kv channels and have different sensitivity to hypoxia. However, our data show that PASMC isolated from the “same” segment of pulmonary arterial tree have different “phenotypes” in terms of (1) the expression level of Kv1.5 (and/or other Kv channel subunits), (2) modulation and regulation of Ca2+ release from IP3-sensitive intracellular stores, and (3) function of a unique oxygen sensor or sensing mechanism intrinsic to PASMC. Different phenotypes of PASMC in rat pulmonary artery seem to determine the sensitivity (or responsiveness and reactivity) to hypoxia. The heterogeneity of PASMC in response to hypoxia or the different sensitivity to hypoxia between hypoxia-sensitive and hypoxia-insensitive PASMC may be generated and regulated by genetic factors.

Physiological significance of heterogeneity of hypoxia-induced changes in I_K(V) and [Ca2+]_cyt in PASMC. Similar to smooth muscle cells in systemic arterial wall (13, 14, 23, 39), PASMC in the pulmonary vasculature are functionally or electrically linked via gap junction channels (70). The gap junction not only enables electrical signals generated in one cell to propagate to others, but also allows [Ca2+]_cyt mobilized from intracellular stores (and other second messengers) in one cell to be carried over to other cells. Therefore, a small group of PASMC or the hypoxia-sensitive cells may function like photoreceptor-containing neurons in retina, pacemaker cells in heart, or pacemaker-like cells in gastrointestinal wall to sense hypoxia, generate action potentials as a result of reduced I_K(V) and subsequent membrane depolarization, induce Ca2+ transients by triggering Ca2+ mobilization, and increase [Ca2+]_cyt. The “local” membrane depolarization and [Ca2+]_cyt increase in hypoxia-sensitive PASMC then pass through the gap junction channels to other PASMC in the arterial segment and ultimately cause pulmonary vasoconstriction.

In summary, PASMC isolated from the same segments of rat pulmonary arterial trees exhibit heterogeneous response to acute hypoxia, by changes in whole-cell I_K(V) and Ca2+ mobilization. The hypoxia-sensitive PASMC manifested by reduction of whole-cell I_K(V) appear to have higher expression level of Kv1.5 than hypoxia-insensitive PASMC in which hypoxia has negligible effect on I_K(V). In hypoxia-sensitive PASMC manifested by Ca2+ mobilization, depletion of IP3-sensitive intracellular stores with CPA abolished the hypoxic effect. The difference of hypoxia-induced effects on whole-cell I_K(V) and Ca2+ release is present in cultured PASMC initially isolated from the same segment of pulmonary arterial tree, indicating that the heterogeneity of PASMC or the heterogeneous response to acute hypoxia is genetically regulated. In other words, a genetically controlled mechanism, or an intrinsically unique mechanism, in certain PASMC can be maintained after cell division and passed into next generations.

The genetic factors that control the mechanism(s) remain incompletely understood. Physiologically, however, it is more efficient that only some cells in the pulmonary vasculature respond to acute hypoxia by changes in Kv channel activity and Ca2+ mobilization. The subsequent membrane depolarization and [Ca2+]_cyt elevation in hypoxia-sensitive PASMC can then propagate to other contractile PASMC via gap junctions and eventually cause a uniformly distributed contraction of the whole segment of pulmonary arteries.

ACKNOWLEDGMENTS

We thank Ann Nicholson for technical assistance. Present address of Y. Yu: Department of Physiology and Biophysics, University of California, Irvine, Irvine, CA.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-054043, HL-064945, and HL-066012. Y. Yu is supported by American Heart Association Scientist Development Grant 0630117N.

REFERENCES


